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The Human Cytomegalovirus UL97 Protein Is Phosphorylated and a Component of Virions

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The expression of the human cytomegalovirus (HCMV) UL97 open reading frame in infected or transfected cells in the presence of the antiherpes compound ganciclovir (GCV) results in the intracellular phosphorylation of GCV. There are conventional kinase domains within the UL97-encoded protein (pUL97). However, the role of pUL97 in the HCMV replication cycle, and the mechanism by which it causes phosphorylation of GCV, are currently unknown. Herein, the biosynthesis and biogenesis of pUL97 was studied in HCMV-infected cells. pUL97 is expressed with early-late kinetics and is posttranslationally modified by phosphorylation. This phosphorylation occurs within 1 hr after synthesis, affects the electrophoretic mobility of pUL97, and is independent of the presence of other HCMV proteins. pUL97 was localized to the nucleus of infected cells and found in the HCMV virions. Thus, pUL97 is a virion phosphoprotein, and a likely tegument component.

INTRODUCTION

Human cytomegalovirus (HCMV) causes significant morbidity and mortality in immunocompromised and immunosuppressed adults, as well as in congenitally infected infants (Britt and Alford, 1996). A common treatment for HCMV disease is the nucleoside analogue ganciclovir (GCV). In HCMV-infected cells, the prodrug GCV is phosphorylated to the active form, GCV triphosphate, a substrate for viral DNA polymerase (UL54), resulting in inhibition of viral DNA synthesis by slowing elongation of viral DNA (Cheng *et al.,* 1983; Biron *et al.,* 1985; Mar *et al.,* 1985; Sullivan *et al.,* 1993; Crumpacker, 1996). HCMV isolates resistant to the inhibitory effect of GCV have mutations in either UL54, affecting the polymerase's affinity for the drug, or in UL97, which encodes a phosphotransferase (Chee *et al.,* 1989; Alain *et al.,* 1993; Littler *et al.,* 1992; Sullivan *et al.,* 1992). UL97 expression in cells confers the ability to phosphorylate GCV in the absence of other HCMV genes (Littler *et al.,* 1992; Metzger *et al.,* 1994). Therefore, UL97 is the only HCMV gene required for GCV phosphorylation.

The UL97 open reading frame encodes a 707-amino acid protein (pUL97) which possesses conventional kinase domains within its C-terminal half (Hanks *et al.,* 1988; Chee *et al.,* 1989). When expressed in bacteria, this C-terminal portion of UL97 is sufficient to cause the phosphorylation of GCV *in vitro* (Littler *et al.,* 1992). pUL97 has amino acid homology with proteins encoded by at least four other herpesviruses, including herpes simplex virus type 1 (HSV-1), Epstein–Barr virus, varicella-zoster virus, and human herpesvirus 6 (Chee *et al.,* 1989; Smith and Smith, 1989). Furthermore, these putative herpesvirus phosphotransferases are within conserved regions of their respective genomes, being similarly positioned relative to genes which encode the major capsid protein, DNA terminase, and alkaline exonuclease (Lawrence *et al.,* 1990). Although this may suggest conservation of function, since their shared homology is limited to the recognized kinase domains of these relatively large proteins (50–80 kDa), Chee *et al.* (1989) caution against the conclusion that these phosphotransferases have the same specificity or function. Notwithstanding, Ng *et al.* (1996) reported recently that UL97 can partially compensate for its putative analog in HSV-1, UL13 (Chee *et al.,* 1989), in recombinant UL13 deletion virus. UL13 is known to encode a protein kinase which phosphorylates an HSV-1 immediate-early protein, ICP22 (α22) (Cunningham *et al.,* 1992; Purves and Roizman, 1992; Purves *et al.,* 1993). By analogy, the function of pUL97 may be to phosphorylate another HCMV-encoded protein, or perhaps a cellular protein, but its physiological substrate has not yet been identified.

Here, we have examined the biosynthesis and biogenesis of pUL97 in HCMV-infected cells. pUL97 is (i) synthesized within a few hours of infection and through late times; (ii) posttranslationally modified by phosphorylation within 1 hr of synthesis; (iii) located in the nuclei of infected cells; and (iv) found in the mature virion. Furthermore, experiments performed with cell lines stably expressing functional pUL97 indicated that phosphorylation of pUL97 was independent of expression of other viral proteins.

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METHODS

DNA sequence

The numbering system of Chee *et al.* (1990) was used for the HCMV strain AD169 DNA sequence (Genbank Accession No. X17403).

Cells and virus

HCMV AD169 was obtained from the American Type Culture Collection and propagated in human foreskin fibroblast (HFF) cells. HFF cells and U373-MG (U373) astrocytoma cells were described (Jones *et al.,* 1995). U373-derived cell lines stably expressing UL97 protein were obtained by transfection of pRcCMVp-UL97 by the calcium phosphate precipitation method, selected in 0.5 μg/ml puromycin, and cloned as described (Kim *et al.,* 1995).

UL97 expression plasmids

The AD169 UL97 gene (nt 140484 to 142604; Chee *et al.,* 1990) was amplified with Taq polymerase (Boehringer Mannheim Biochemicals, Indianapolis, IN) from the AD169 *Hin*dIII-S fragment (pHindS; Oram *et al.,* 1982) with the following primers: 5′GGAATTCATATGTCCTCCGCACTTCGGTC3′ and 5′GGAATTCTTACTCGGGGAACAGTTGGC3′, introducing a *Nde*I site at the initiating ATG and an *Eco*RI site at both ends of the gene, and cloned into pUC118. The internal *Asc*I fragment (nt 140577–141996) was replaced by the corresponding *Asc*I fragment from pHindS and the remaining nucleotides were confirmed by sequencing. The UL97 gene was then cloned as a *Nde*I–*Eco*RI fragment into the prokaryotic expression vector, pT7K (Plotch *et al.,* 1989), to yield pT7K-UL97. For eukaryotic expression, UL97 was placed under the control of the immediate-early HCMV enhancer-promoter in pRcCMVp-UL97. Briefly, the UL97-containing *Nde*I–*Eco*RI fragment, along with a linker which provided a favorable eukaryotic consensus translation sequence (Kozak, 1991) (5′AGCTTGGTACCGAGCTCGACGCCGCCAC3′ and 5′TAGTGGCGGCGTCGACGAGCTCGGTACCA3′) were cloned into *Hin*dIII/*Eco*RI-digested pRcCMVp. pRcCMVp was derived from pRcCMV (Invitrogen, San Diego, CA) by replacing the neomycin-resistance gene with the puromycin-resistance gene (de la Luna and Ortin, 1992).

Antibodies

UL97 synthesis in *Escherichia coli* strain BL21(λDE3)-LysS harboring pT7K-UL97 was induced with 1 m*M* isopropyl-β-D-thiogalactopyranoside for 3 hr. After sonication to lyse the bacterial cells, protein extracts from the pellet fraction were subjected to SDS–PAGE. The 80-kDa band corresponding to UL97 protein was cut from the gel, eluted, and used to immunize female New Zealand white rabbits (Pocono Rabbit Farm, Canadensis, PA). Production of antiserum BX49 against the HCMV UL80-encoded assembly protein was described (Jones *et al.,* 1994).

Protein analysis

Metabolic labeling with [^{35}S]methionine/cysteine, immunoprecipitation, and immunoblot techniques were performed as described (Jones *et al.,* 1994; 1995). For radiolabeling experiments involving nonradioactive chases, radioactive media was removed and replaced with nonradioactive complete media after two washes with PBS; incubation was continued until the proper harvest time. For phosphate labeling, cells grown on 60-mm dishes were washed in phosphate-free media and labeled with 200 μCi [^{32}P]orthophosphate per milliliter (NEN DuPont, Cambridge, MA) in phosphate-free media (Gibco, Gaithersburg, MD). Cells were then washed twice in PBS and processed as described for [^{35}S]methionine/cysteine-labeled cells (Jones *et al.,* 1994). For phosphatase treatment, immunoprecipitates were washed in 50 m*M* Tris–HCl, pH 7.8, 5 m*M* DTT, 2 m*M* $MgCl_2$, 0.1 mg/ml BSA, and treated with 800 U lambda-phosphatase (New England Biolabs, Beverly, MA) for 30 min at 30° in the same buffer.

Virion purification

The media from a 150-cm^2 flask of HFF cells infected with HCMV at a multiplicity of infection of 5 PFU/cell, was collected 5 days after infection, cleared by centrifugation for 10 min at 3,000 *g* at 4°, and subjected to two rounds of purification over tartrate-glycerol gradients as described (Talbot and Almeida, 1977; Irmiere and Gibson, 1983). Bands corresponding to noninfectious enveloped particles and virion fractions were collected from the centrifuge tubes by side puncture after visualization by light-scattering. Corresponding fractions from 5 gradients following an initial centrifugation (15 min) were pooled and applied to a new glycerol-tartrate gradient and recentrifuged for 18 hr. Bands were collected similarly, diluted to 8 ml with 40 m*M* sodium phosphate (pH 7.4)/100 m*M* NaCl, and pelleted at 85,000 *g* for 90 min at 4°. Pellets were resuspended in SDS-gel buffer (Sambrook *et al.,* 1989) and boiled prior to SDS–PAGE.

Immunofluorescence

Uninfected and HCMV-infected (multiplicity of infection of 5 PFU/cell) HFF cells were fixed at 3 days p.i., permeabilized, and treated with antibodies as described (Kim *et al.,* 1995), except that the primary antibody was diluted 1:100, and the secondary antibody was 1:250-diluted FITC-conjugated donkey-anti-rabbit immunoglobulin G (Pierce, Rockford, IL).

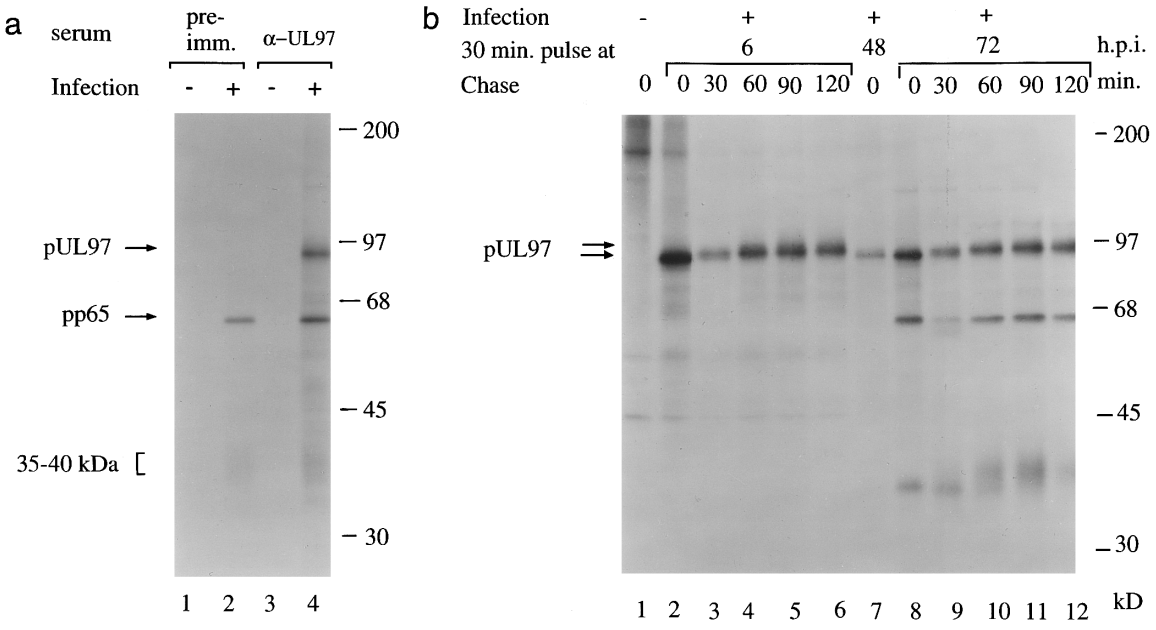

FIG. 1. Metabolic labeling of pUL97. (a) Uninfected (lanes 1 and 3) or HCMV-infected (lanes 2 and 4) HFF cells were labeled at 3 days p.i. for 4 hr with [^{35}S]methionine/cysteine. Cells were lysed and either preimmune serum (lanes 1 and 2) or UL97 antiserum (lanes 3 and 4) was added, followed by protein A–Sepharose. Samples were then analyzed by SDS–PAGE followed by autoradiography. The migration of pUL97, pp65, and an unknown protein of 35–40 kDa are indicated. The positions of molecular weight markers are indicated in kDa. (b) Uninfected (lane 1) or HCMV-infected (lanes 2–12) HFF cells were pulse-labeled at the indicated times p.i. for 30 min with [^{35}S]methionine/cysteine and chased with excess unlabeled methionine and cysteine for 0 (lanes 1, 2, 7, and 8), 30 (lanes 3 and 9), 60 (lanes 4 and 10), 90 (lanes 5 and 11), or 120 min (lanes 6 and 12). Cells were lysed and pUL97 immunoprecipitated using UL97 antiserum. Samples were then analyzed by SDS–PAGE followed by autoradiography. Arrows indicate the range of migration of pUL97.

RESULTS

UL97 antiserum

The HCMV AD169 UL97 gene was cloned in a prokaryotic expression vector and expressed in *E. coli;* pUL97 was excised from a SDS–PAGE gel and injected into a rabbit for antibody production. The antiserum was tested for specificity by immunoprecipitation using radiolabeled lysates from HCMV-infected and uninfected HFF cells (Fig. 1a). Besides the expected band corresponding to pUL97, two additional proteins were observed in immunoprecipitations using late infected-cell lysates (lane 4). These proteins are observed when using all rabbit antisera (data not shown) and are nonspecifically precipitated, as evidenced by their precipitation with preimmune serum (lanes 1 and 2) and with normal human serum (data not shown). The protein of approximately 65 kDa is believed to be pp65, an abundant HCMV late protein. The nature of the 35- to 40-kDa protein is unknown. The ~80-kDa UL97 protein was the only protein uniquely precipitated by the immune serum (lane 4), demonstrating the specificity of the UL97 antiserum.

Expression and virion localization of pUL97

Protein expression from UL97 was analyzed using polyclonal antiserum obtained from rabbits immunized with pUL97 expressed in *E. coli.* The accumulation of pUL97 in HCMV-infected cells was analyzed by immunoblotting, using cell lysates taken at different times after infection (Fig. 2a). At 5 hr p.i. pUL97 was readily detected; further accumulation of pUL97 occurred steadily through late times postinfection. Since trace levels of pUL97 were present at 2–4 hr p.i., but not in uninfected cells (Figs. 2a and 2b), viral particles twice purified on glycerol-tartrate gradients were analyzed for the presence of pUL97 (Fig. 2c). pUL97 protein was detected both in noninfectious enveloped particles (Irmiere and Gibson, 1983; 1985) and virions, which comigrated with pUL97 from infected cells. Thus, the data from Fig. 2, as well as results of metabolic radiolabeling-immunoprecipitation experiments (see below), indicate that pUL97 is synthesized throughout most of the replication cycle and is a virion constituent. Furthermore, the trace levels of pUL97 detected at 2–4 hr p.i. represent protein which entered the cell with the virion and not *de novo* protein synthesis, since similar levels of pUL97 were detected in the presence of protein synthesis inhibitors (data not shown). Pulse–chase experiments at both early and late times postinfection indicated that pUL97 is stable, with no detectable turnover after an 8-hr chase (data not shown).

Intracellular localization

Hydropathy analysis of UL97 protein does not predict any hydrophobic domains large enough to provide a

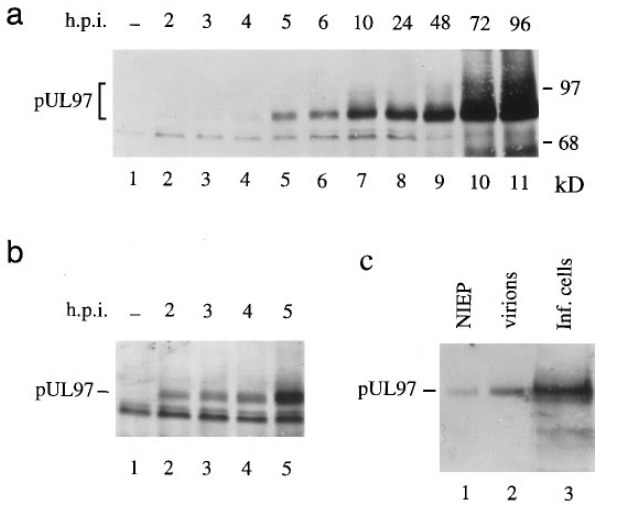

FIG. 2. pUL97 expression in infected cells and purified virions. (a) Uninfected (lane 1) or HCMV-infected HFF cells lysed at 2, 3, 4, 5, 6, 10, 24, 48, 72, or 96 hr p.i. (lanes 2–11) were subjected to SDS–PAGE and analyzed by immunoblotting with UL97 antiserum. The location of pUL97, which has a heterogeneous mobility, is indicated. (b) A darker exposure of part of panel a, demonstrating the presence of pUL97 at 2–4 hr p.i. (c) Noninfectious enveloped particles (NIEPs; lane 1) and virions (lane 2) were purified twice by centrifugation through glycerol–tartrate gradients and analyzed by Western blotting. A lysate from HCMV-infected HFF cells (72 hr p.i.) was loaded for comparison (lane 3).

transmembrane domain (data not shown). Since pUL97 is present in virions, it is probably located in the tegument or capsid, which assemble in the nucleus of infected cells (Roizman and Sears, 1993; Ward *et al.,* 1996). Therefore, pUL97 is predicted to localize to the nucleus. This prediction was confirmed by indirect immunofluorescence using UL97 antiserum (Fig. 3). Although cytoplasmic fluorescence of infected cells by UL97 antiserum was slightly increased relative to that observed with preimmune serum (Figs. 3a and 3b), pUL97 localized predominantly to the nucleus (Fig. 3b).

Posttranslational modification

It was observed from immunoblot analysis that pUL97 migrates heterogeneously in SDS–PAGE; this is especially evident at late times p.i. when relatively abundant steady-state levels of pUL97 are present (Fig. 2a, lanes 10 and 11). Although the data in Fig. 2 were from laboratory strain AD169-infected cells, identical results concerning pUL97 abundance and heterogeneity were obtained in experiments utilizing clinical isolates of HCMV (data not shown). To investigate whether this heterogeneity was due to posttranslational modification, pUL97 was examined in a pulse–chase radiolabeling-immunoprecipitation experiment (Fig. 1b). HCMV-infected HFF cells were pulse-labeled for 30 min with [^{35}S]methionine/cysteine at different times p.i. and chased in the presence of unlabeled methionine and cysteine for 30, 60, 90, or 120 min followed by immunoprecipitation with UL97 antiserum (Fig. 1b). pUL97 pulse-labeled at both early (6 hr p.i.; lane 2) and late (48 and 72 hr p.i.; lanes 7 and 8) times p.i. migrated as a single band of approximately 80 kDa, but its electrophoretic mobility was substantially retarded after a 60-min or longer chase (lanes 4–6 and 10–12). The electrophoretic mobility of pUL97 was retarded to an intermediate level after a 30-min chase

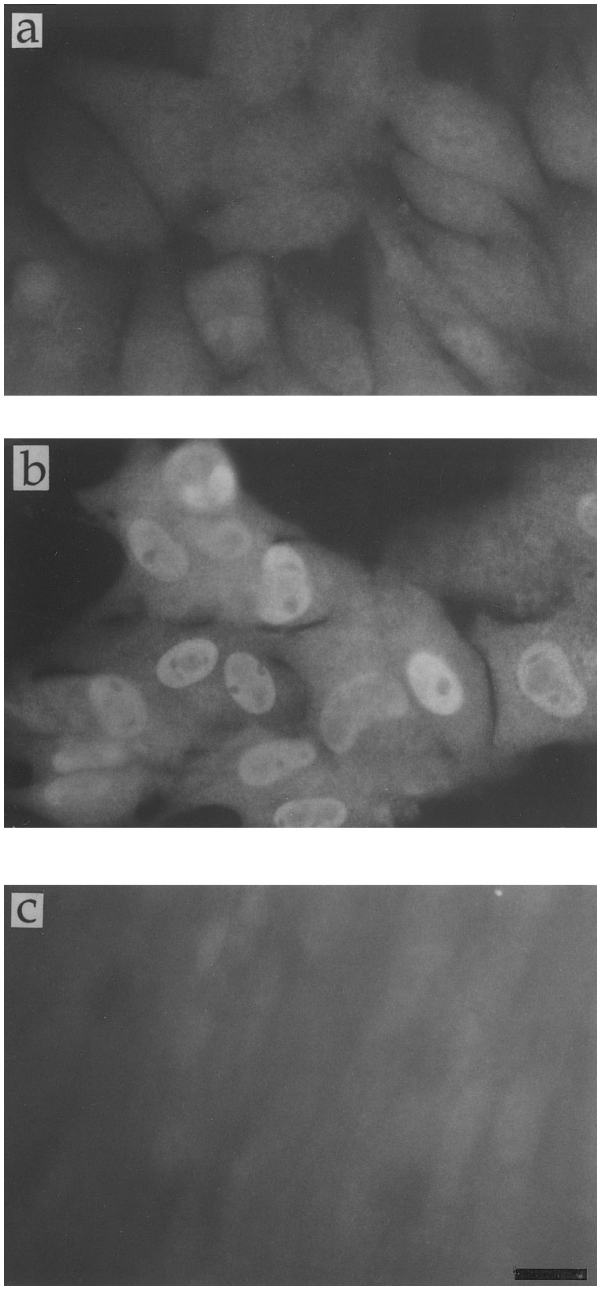

FIG. 3. Subcellular localization of pUL97. HCMV-infected HFF cells at 3 days p.i. (a and b) or uninfected HFF cells (c) were analyzed by indirect immunofluorescence using either preimmune serum (a) or UL97-specific antiserum (b and c). Prior to incubation with rabbit sera, cells were exposed to normal human serum to eliminate detection of nonspecific binding of rabbit immunoglobulins to uninfected or infected cell proteins. Bar is 25 μm.

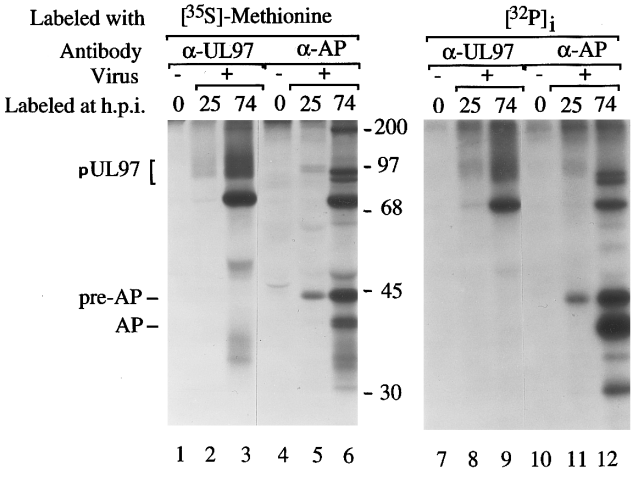

FIG. 4. pUL97 is phosphorylated. Uninfected ("−") or infected HFF cells ("+") were labeled for 4 hr with [^{35}S]methionine/cysteine (lanes 1–6) or for 16 hr with [^{32}P]$_i$ (lanes 7–12) and lysed at indicated times postinfection. pUL97 (lanes 1–3 and 7–9) or assembly protein (AP; lanes 4–6, and 10–12) were immunoprecipitated with the respective antisera and analyzed by SDS–PAGE followed by autoradiography. The anti-AP antiserum (BX49) precipitates bands of 80 and 85 kDa, both protease-precursors (Jones *et al.,* 1994), and 44 (pre-AP) and 37 kDa (AP). The positions of pUL97 and pre-AP and AP are indicated. The band at approximately 68 kDa in lanes 3, 6, 9, and 12 is a nonspecifically immunoprecipitated late viral phosphoprotein, probably pp65.

(lanes 3 and 9). Thus, pUL97 is posttranslationally modified within 1 hr after synthesis.

pUL97 is phosphorylated

To investigate the nature of the apparent molecular weight increase, pUL97 was examined for the presence of glycosylation and phosphorylation. There was no indication that pUL97 was glycosylated, since we were unable to detect incorporation of radiolabeled galactose or glucose into pUL97 (data not shown). Possible phosphorylation of pUL97 was examined by metabolic labeling of HCMV-infected HFF cells with [^{32}P]$_i$ at 25 or 74 hr p.i. followed by immunoprecipitation with UL97 antiserum (Fig. 4). As a positive control, UL80-encoded proteins containing the C-terminal assembly protein domain were immunoprecipitated with BX49 antiserum (Jones *et al.,* 1994). In addition to the 80- and 85-kDa protease precursor proteins, the 44-kDa assembly protein precursor and the 37-kDa mature assembly protein are recognized by antiserum BX49. As shown in Fig. 4 (lanes 10–12), all assembly protein species were phosphorylated, as shown previously (Roby and Gibson, 1986). pUL97 was likewise labeled with [^{32}P]$_i$, and continued to migrate heterogeneously (Fig. 4, lanes 8 and 9). As further evidence that pUL97 is a phosphoprotein ^{32}P-labeled pUL97 was treated with lambda-phosphatase, which removed all label from the protein (data not shown).

pUL97 was digested with lambda-phosphatase to determine whether phosphorylation was responsible for the electrophoretic mobility shift we observed within 1 hr after synthesis. pUL97 metabolically labeled with [^{35}S]methionine/cysteine was immunoprecipitated from HCMV-infected cells and treated with lambda-phosphatase (under conditions which should remove phosphate on serine, threonine, and tyrosine residues), and analyzed by SDS–PAGE (Fig. 5). pUL97 continuously labeled for 4 hr, or pulse-labeled for 30 min and chased for 90 min, had an increased electrophoretic mobility after phosphatase treatment (lanes 2 and 6) as compared to the corresponding untreated samples (lanes 1 and 5); however, they had an electrophoretic mobility equal to that of the untreated 30-min pulse samples (lanes 3 and 7). Phosphatase treatment of the 30-min pulse sample did not alter the electrophoretic mobility of pUL97 (lanes 4 and 8). Taken together, the data indicate that within the 30-min pulse, pUL97 did not acquire phosphate yet. However, by 1 hr after the pulse, pUL97 became phosphorylated resulting in decreased electrophoretic mobility relative to unphosphorylated pUL97. Other phosphatases were also used (protein-phosphatase 1, 2A, and 2B) but, at best, resulted in only partial dephosphorylation of pUL97 (data not shown).

Phosphorylation of pUL97 does not require other viral proteins

pUL97 was examined in the absence of other viral proteins by construction of U373-derived cell lines which constitutively express UL97 under the control of the HCMV major immediate-early enhancer-promoter. Multiple cell lines were obtained, two of which, 97-7 and 97-8, were studied (Fig. 6). Immunoblot analysis revealed a UL97-reactive protein which comigrated with pUL97 obtained from CMV-infected cells (Fig. 6a). This protein was not observed in cell lines obtained after transfection with the empty vector (i.e., cell line Rcp). pUL97 expressed in 97-7 and 97-8 cell lines was shown to be

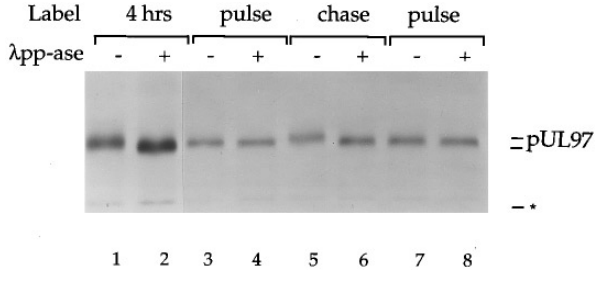

FIG. 5. Treatment with lambda-phosphatase removes phosphate from pUL97. pUL97 immunoprecipitated from HCMV-infected HFF cells, which were previously labeled at 72 hr p.i. for 4 hr (lanes 1 and 2) or pulse-labeled for 30 min (lanes 3, 4, 7, and 8) with [^{35}S]methionine/cysteine followed by a 120-min chase (lanes 5 and 6), were treated with lambda-phosphatase ("+") or mock-treated ("−"). The positions of the phosphorylated (top band) or dephosphorylated (bottom-band) forms of pUL97 are indicated. A nonspecifically precipitated late viral protein is indicated (*).

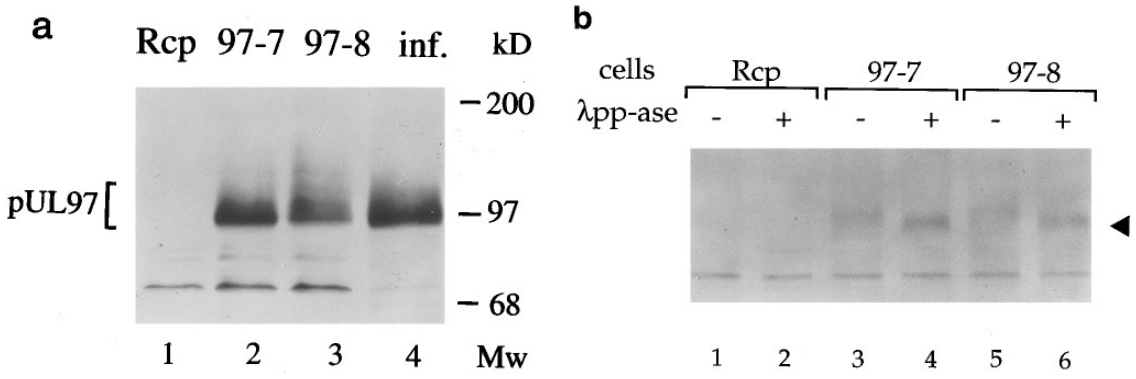

FIG. 6. Phosphorylation of pUL97 does not require other viral proteins. (a) Immunoblot analysis of proteins from U373-derived cell lines stably expressing pUL97 (97-7 and 97-8, lanes 2 and 3), empty vector control cells (Rcp, lane 1) together with late HCMV-infected HFF cells (72 hr p.i.; lane 4). (b) Rcp, 97-7, and 97-8 cell lines were labeled for 16 hr with [^{35}S]methionine/cysteine, pUL97 was immunoprecipitated and treated with lambda-phosphatase ("+" ; lanes 2, 4, and 6) or mock-treated ("−" ; lanes 1, 3, and 5). Immunoprecipitates were then analyzed by SDS–PAGE followed by autoradiography. The arrowhead indicates the dephosphorylated form of pUL97, which migrates slightly faster than the phosphorylated form.

functional since growth of a thymidine kinase negative (tk$^-$) mutant of HSV-1 (Jones *et al.,* 1991) in these cells was inhibited in the presence of GCV; there was no inhibition of HSV-1 tk$^-$ virus growth in the presence of GCV in Rcp cells (data not shown). Similar to HCMV-infected cells, pUL97 immunoprecipitated from 97-7 and 97-8 cells, after metabolic labeling with [^{32}P]$_i$, contained labeled phosphate (data not shown). In addition, the migration of pUL97 in SDS–PAGE after phosphatase treatment (Fig. 6b) was examined. Like pUL97 from HCMV-infected cells, pUL97 from the cell lines was sensitive to treatment with lambda-phosphatase, as indicated by its increased electrophoretic mobility (lanes 4 and 6). These results indicate that phosphorylation of pUL97 does not require the presence of other HCMV gene products and is therefore the result either of phosphorylation by cellular kinases or of autophosphorylation.

DISCUSSION

The HCMV UL97 gene plays a central role in conferring sensitivity to GCV since it is responsible for the initial phosphorylation of GCV to GCV-monophosphate (GCV-MP) which leads to the full phosphorylation of GCV-MP by cellular nucleoside kinases to the active GCV-triphosphate (Biron *et al.,* 1985; Littler *et al.,* 1992; Sullivan *et al.,* 1992). The role of the UL97 gene in viral replication, however, is not clear. It is thought that UL97 plays an essential role in viral replication based on the following: (i) UL97 has homology to genes of other herpesviruses and is located within the conserved region of herpesvirus genomes containing capsid morphogenesis and DNA processing functions (Chee *et al.,* 1989; Lawrence *et al.,* 1990); (ii) efforts to isolate a virus with a deletion of the UL97 gene have failed (Michel *et al.,* 1996), and (iii) GCV-resistant HCMV isolates with mutations in the UL97 gene contain point mutations or, at most, small (four amino acids) deletions within domains not conserved among protein kinases (Chou *et al.,* 1995; Sullivan *et al.,* 1992). This is in contrast with the HSV-1 thymidine kinase gene, which is responsible for the phosphorylation of GCV and acyclovir (Fyfe *et al.,* 1978), but is not essential for virus growth in tissue culture (Efstathiou *et al.,* 1989). As a prelude to studies concerning the physiological role of UL97 in HCMV replication, the expression of UL97 in HCMV-infected cells was examined. Herein, we report that pUL97 is synthesized throughout most of the replicative cycle, is posttranslationally modified by phosphorylation, and is a component of HCMV virions.

Our initial observation that pUL97 was posttranslationally modified was due to its heterogeneous electrophoretic mobility (Fig. 2). Several proteins are affected in their mobility in SDS–PAGE by phosphorylation. The SV40 large T antigen is phosphorylated on many residues, but only the phosphorylation of threonine 124 or 701 increases its electrophoretic mobility (Grässer and Konig, 1992). In contrast, the EBNA 2A protein of Epstein–Barr virus shows a decrease in mobility upon phosphorylation (Grässer *et al.,* 1991). We found that within 1 hr after synthesis all pUL97 was phosphorylated and this phosphorylation was completely responsible for the observed decrease in electrophoretic mobility of the protein. Phosphorylation of pUL97 also occurred in cells that expressed it in the absence of other viral proteins, which indicated that pUL97 was phosphorylated either by a cellular kinase or by autophosphorylation (Fig. 6). He *et al.* (1996) recently reported the *in vitro* autophosphorylation of recombinant pUL97, produced in insect cells, on serine and threonine residues. These authors reported that inactivation of enzymatic activity by mutation of an active site residue did not completely abolish the incorporation of radioactive phosphate into pUL97, indicating that cellular kinases may also contribute to the total phosphorylation of pUL97. Lambda-phosphatase re-

moves phosphate from serines and threonines and, at higher concentrations of enzyme, also from tyrosine residues. Here we used lambda-phosphatase in amounts sufficient to remove phosphate from serine, threonine, and tyrosine residues. Other phosphatases, with more limited substrate specificity, were unable to completely remove phosphate from pUL97, indicating either that the phosphate(s) on pUL97 were not accessible to these phosphatases or that the amino acid sequence surrounding the phosphorylation site do not conform to the recognition specificity of these phosphatases.

De novo synthesis of pUL97 begins at 5 hr p.i. and continues through the remainder of the replicative cycle, with relatively high steady state levels of pUL97 at late times p.i. (Fig. 2). The appearance of newly synthesized pUL97 was in agreement with the onset of UL97 transcription as reported by Wing and Huang (1995), consistent with the grouping of UL97 in the class of early-late genes. In contrast to data reported by Michel *et al.* (1996) who detected pUL97 in HCMV-infected cells beginning at 16 hr p.i. by immunoblotting, pUL97 synthesis was detected as early as 5 hr p.i. in our experiments. This apparent discrepancy could be explained by qualitative and quantitative differences in the antibodies used. Trace levels of pUL97 were detected in infected cells prior to 5 hr p.i., consistent with the presence of pUL97 in mature virions. Further experiments have revealed that pUL97 detected from 2 to 4 hr p.i. entered the cells as a component of the infecting virions (data not shown).

pUL97 is likely to be localized to the tegument of the HCMV virion, since hydropathy analysis of pUL97 does not reveal any region likely to be a transmembrane domain, and pUL97 does not correspond to the HCMV analog of known herpesvirus capsid proteins (Gibson end Roizman, 1972; Cohen *et al.,* 1980; Chee *et al.,* 1990; Rixon *et al.,* 1990). The localization of pUL97 in the nucleus of infected cells, reported here and recently by Michel *et al.* (1996), is also consistent with inclusion in the virion tegument, since the herpesvirus tegument proteins are acquired in the nucleus (Roizman and Sears, 1993; Ward *et al.,* 1996). Roby and Gibson (1986) reported the presence of an 80-kDa phosphoprotein in HCMV virions. We have found that pUL97 is a virion phosphoprotein with the appropriate molecular weight and is therefore a candidate for that unassigned 80-kDa protein. If so, the abundance of the 80-kDa protein in dense bodies (Roby and Gibson, 1986) suggests that pUL97 is located in the tegument. Furthermore, pUL97 is likely to be a minor constituent of HCMV virions since (i) the 80-kDa phosphoprotein was detected in virions by metabolic labeling with $[^{32}P]_i$, but not with $[^{35}S]$methionine (Roby and Gibson, 1986); and (ii) it was not detected by direct amino acid sequence analysis of virion proteins (Baldick and Shenk, 1996), a procedure which may miss minor virion constituents.

As a virion phosphoprotein which becomes a stable component of newly infected cells, pUL97 may perform an important role during the immediate-early phase of HCMV replication. For example, the HSV-1 UL13 kinase, a homologue of UL97 (Chee *et al.,* 1989), is expressed abundantly at late times and is a component of the HSV-1 virions (Overton *et al.,* 1992). ICP22, an immediate-early protein, has been identified as a substrate of UL13 protein kinase (Purves and Roizman, 1992). The phosphorylation of ICP22 is important for replication of HSV-1 in rodent cell lines (Purves and Roizman, 1992; Purves *et al.,* 1993). Another HCMV virion component, the pp65 tegument phosphoprotein, which is a presumed kinase (Britt and Auger, 1986, Michelson *et al.,* 1984), but is nonessential for HCMV replication (Schmolke *et al.,* 1995), has a role at immediate-early times after infection. This phosphoprotein, encoded by UL83 with early-late expression kinetics (Depto and Stenberg, 1989), is introduced into cells by the infecting virions and functions at immediate-early times to eliminate presentation of the immediate-early IE1 protein at the cell surface by causing the phosphorylation of that protein (Gilbert *et al.,* 1996). The HCMV tegument phosphoprotein pp71, encoded by UL82, has been recognized as playing an important role in the transcriptional stimulation of immediate-early genes (Liu and Stinski, 1992). The above-mentioned tegument proteins all have important functions in viral replication; it is conceivable that pUL97 also has such a regulatory role. Consistent with an important role in the infection cycle is that UL97 deletion mutants could not be purified (Michel *et al.,* 1996). Thus, elucidation of the function of pUL97 in the HCMV replication cycle may depend on the generation of UL97 conditional lethal or host range viral mutants (Kim *et al.,* 1995; Compton, 1993). The degree to which phosphorylation of pUL97 may impact its function can be elucidated once its physiological target is identified.

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